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Functional incorporation of beef-heart cytochrome *c* oxidase into membranes of *Streptococcus cremoris*

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Beef heart mitochondrial cytochrome *c* oxidase has been incorporated into membrane vesicles derived from the homofermentative lactic acid bacterium *Streptococcus cremoris*. Proteoliposomes containing cytochrome *c* oxidase were fused with the bacterial membrane vesicles by means of a freeze/thaw sonication technique. Evidence that membrane fusion has taken place is presented by the demonstration that nonexchangeable fluorescent phospholipid probes, originally present only in the bacterial membrane or only in the liposomal membrane, are diluted in the membrane after fusion and, by sucrose gradient centrifugation, indicating a buoyant density of the membranes after fusion in between those of the starting membrane preparations. The fused membranes are endowed with a relatively low ion permeability which makes it possible to generate a high proton motive force (100 mV, inside negative and alkaline) by cytochrome-*c*-oxidase-mediated oxidation of the electron donor system ascorbate/*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine/cytochrome *c*. In the fused membranes this proton motive force can drive the uptake of several amino acids via secondary transport systems.

The incorporation procedure described for primary proton pumps in biological membranes opens attractive possibilities for studies of proton-motive-force-dependent processes in isolated membrane vesicles from bacterial or eukaryotic origin which lack a suitable proton-motive-force-generating system.

Isolated bacterial membrane vesicles are excellent model systems for studies on the role of the proton motive force (Δp) on solute transport if a suitable Δp-generating system is present [1, 2].

The only Δp-generating system present in membrane vesicles of many fermentative bacteria is the proton-translocating ATPase [3]. The localization of the active site of this enzyme on the inner side of the membrane vesicles excludes ATP hydrolysis as a generating mechanism. In order to study solute transport in these membrane vesicles, procedures have been developed to generate artificially a membrane potential ($\Delta\psi$) [4] and/or transmembrane pH gradient (ΔpH) [5]. These methods are based on ionophore-mediated translocation of potassium ions or the translocation of weak acids or bases in response to their own chemical gradients. A $\Delta\psi$ or ΔpH of considerable magnitude can be generated with these procedures. However, the transient character of these potentials severely limits the application of these procedures for studies on solute transport. Therefore model system(s) are needed in which a Δp can be generated for longer periods of time.

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Abbreviations. Ph_4P^+, tetraphenylphosphonium ion; $Ph(NMe_2)_2$, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Δp, proton motive force; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane proton gradient; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine β-sulfonyl)phosphatidylethanolamine; R_{18}, octadecyl rhodamine β-chloride; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitro-salicylanilide.

Enzyme. Cytochrome *c* oxidase or ferrocytochrome *c*: oxygen oxidoreductase (EC 1.9.3.1).

The incorporation of Δp-generating systems into liposomes have been performed successfully [6–9]. Since these proteoliposomes are endowed with a low proton permeability, a Δp of considerable magnitude can be generated when an appropriate energy source is provided. Recently, it has been shown that bacterial membrane vesicles can be fused easily with (proteo)liposomes by various procedures [10, 11]. Combination of both procedures has led to a functional incorporation of the light-induced proton pump bacteriorhodopsin into membrane vesicles of the homofermentative lactic acid bacterium *Streptococcus cremoris* [12]. The orientation of bacteriorhodopsin was such that upon illumination a reversed Δp ($\Delta\psi$, interior positive, and ΔpH, interior acid) was generated which makes this system only suitable for studies of solute extrusion systems. Therefore we have searched for a Δp-generating system which can be selectively manipulated in such a way that a right-side-out Δp ($\Delta\psi$, interior negative, and ΔpH, interior alkaline) can be generated in the fused membranes. The system of choice was beef heart cytochrome *c* oxidase. This enzyme catalyzes the reduction of molecular oxygen to water, the terminal reaction in the mitochondrial electron transport chain. It can be isolated, purified and reconstituted into proteoliposomes [6, 13].

In this paper we demonstrate that *S. cremoris* membrane vesicles can be fused effectively by a freeze/thaw sonication technique with proteoliposomes containing the beef heart mitochondrial cytochrome *c* oxidase. With the membrane-impermeant electron donor reduced cytochrome *c*, a right-side-out Δp of considerable magnitude can be selectively generated in the fused membranes. In this fused membrane system amino acid transport can be energized at high rates upon oxidation of reduced cytochrome *c*. Initial observations

with this fused membrane system have been published previously [14].

MATERIALS AND METHODS

Growth of S. cremoris *and isolation of membrane vesicles*

Streptococcus cremoris Wg2 (prt$^-$) was grown anaerobically on MRS broth at a controlled pH of 6.4 in a 5-l fermenter. Membrane vesicles of *S. cremoris* were prepared as described previously [15], suspended in 50 mM potassium phosphate pH 7.0 containing 10 mM $MgSO_4$ at a concentration of 10–15 mg protein/ml and stored in liquid nitrogen.

Incorporation of cytochrome c *oxidase in liposomes*

Cytochrome *c* oxidase was isolated from beef heart as described by Yu et al. [16]. The heme content was determined using an absorption coefficient of 13.5 $mM^{-1} \cdot cm^{-1}$ for reduced-minus-oxidized heme at the wavelength couple 605 nm/630 nm and was found to be 10.4 nmol/mg protein. For incorporation of cytochrome *c* oxidase in liposomes, 40 mg acetone-washed asolectin and 18 mg octyl β-D-glucopyranoside in 2 ml of 10 mM Hepes/KOH, pH 7.0 containing 45 mM KCl were cosonicated till clarity with an MSE probe-type sonicator on ice under a constant stream of nitrogen gas. Cytochrome *c* oxidase (9 nmol heme *a*) was added and the mixture was dialyzed against 500 vol. of 10 mM Hepes/KOH pH 7.0, supplemented with 45 mM KCl. Subsequently dialysis was continued against 500 vol. of the same buffer supplemented with 5 mM $CaCl_2$. Excess calcium was removed by dialysis overnight against 500 vol. buffer without $CaCl_2$. Liposomes obtained with this procedure had a diameter of 50–300 nm. The respiratory control index was 2.5–3.0 [16].

Fusion of proteoliposomes with S. cremoris *membrane vesicles*

S. cremoris membrane vesicles (75 µl), containing 1.2 mg protein, and cytochrome *c* oxidase proteoliposomes (500 µl), containing 2.25 nmol heme *a*, were mixed and rapidly frozen in liquid nitrogen. Subsequently the mixture was slowly thawed for 20 min at room temperature and the resulting turbid suspension was vortexed and sonicated for 8 s in a plastic tube with an MSE probe-type sonicator.

Sucrose density gradient centrifugation

Centrifugal analysis of the membrane before and after fusion was carried out using a stepwise sucrose gradient. Sucrose was dissolved to different concentrations in 100 mM KCl, 1 mM EDTA and 10 mM Tricine/KOH (pH 8.0). Sucrose concentrations (w/w) of 15% (1.5 ml), 30% (1 ml), 34% (1 ml), 38% (1 ml), 42% (1 ml) and 46% (1 ml) were layered on a 65% sucrose cushion in Beckman SW41 tubes. *S. cremoris* membrane vesicles (0.5 ml) containing 0.52 mg membrane protein, cytochrome *c* oxidase proteoliposomes (0.04 nmol oxidase) and membranes obtained after fusion of mixtures of these membrane preparations were layered on the gradients and centrifuged at 145000 × *g* for 19 h at 18 °C. The tubes were fractionated (0.5 ml) and assayed for *S. cremoris* membrane protein [18], liposomal phospholipid and refractive index. The liposomal phospholipid content was estimated from the fluorescence of NBD-PE [19], originally incorporated into the cytochrome *c* oxidase proteoliposomes at a concentration of 0.5 mol/100 mol phospholipid phosphorus.

Fusion assays

The resonance energy transfer fusion assay was performed essentially as described by Struck et al. [19]. Cytochrome *c* oxidase was incorporated into liposomes by the detergent dialysis method as described above, except that 0.6 mol/100 mol each of the fluorescent donor (NBD-PE) and acceptor (Rh-PE) phospholipid were added to asolectin. At this probe concentration the decrease of energy transfer efficiency as a result of fusion is approximately linearly related to the dilution factor [10, 19]. Cytochrome *c* oxidase proteoliposomes (50 nmol phospholipid phosphorus) were fused with 5–250 nmol phospholipid phosphorus of nonlabeled *S. cremoris* membrane vesicles in a final volume of 100 µl. NBD-PE fluorescence was determined prior and after the addition of 1% (v/v) Triton X-100. NBD-PE fluorescence was corrected for sample dilution, light scatter and the effect of Triton X-100 on the quantum yield of NBD-PE fluorescence as described [19]. Excitation and emission were performed at 475 nm and 530 nm, respectively.

The R_{18} fusion assay was performed as described by Hoekstra et al. [20]. *S. cremoris* membrane vesicles were labeled with R_{18} essentially as described for viral membranes [20]. A solution of 200 nmol R_{18} in 20 µl ethanol was added under extensive vortexing to a 2-ml suspension of *S. cremoris* membrane vesicles, containing 50 µmol phospholipid phosphorus. The mixture was incubated in the dark for 1 h at room temperature. Non-incorporated R_{18} was removed by chromatography of the membrane suspension over a Sephadex G-75 column (1 × 20 cm). The labeled membranes, eluting in the void volume, were washed twice with 10 mM Hepes/KOH pH 7.0 containing 35 mM KCl. At the probe concentration used (4 mol/100 mol phospholipid phosphorus), a linear relationship exists between the efficiency of self-quenching and the ratio of R_{18} to total phospholipid [20]. The extent of fusion is therefore directly proportional to the extent of R_{18} fluorescence. R_{18} (14 nmol phospholipid phosphorus) labeled *S. cremoris* membrane vesicles were fused with 7–140 nmol phospholipid phosphorus nonlabeled cytochrome *c* oxidase proteoliposomes in a final volume of 100 µl. R_{18} fluorescence was determined prior and after the addition of 1% (v/v) Triton X-100 as described [20]. Excitation and emission were performed at 560 nm and 590 nm, respectively. Fluorescence was determined using a Perkin-Elmer MPF4 spectrofluorimeter.

Determination of $\Delta\psi$ *and* ΔpH

$\Delta\psi$ (interior negative) was determined from the distribution of tetraphenylphosphonium (Ph_4P^+) across the membrane using a Ph_4P^+ selective electrode [21]. Reaction mixtures contained (final concentrations) 10 mM Hepes/KOH pH 7.0, 45 mM KCl, 5 mM $MgCl_2$ and 2 µM Ph_4P^+ in a total volume of 1 ml. Membranes were added as indicated followed by the addition of 20 µM cytochrome *c* and 10 mM ascorbate, unless otherwise indicated. $Ph(NMe_2)_2$, nigericin and valinomycin were used at final concentrations of 400 µM, 10 nM and 2 µM, unless otherwise indicated. The internal Ph_4P^+ concentration was determined from the amount of Ph_4P^+ which disappeared from the external medium and $\Delta\psi$ was calculated with the Nernst equation. A correction for

concentration-dependent binding of Ph_4P^+ to the membranes, according to the model of Lolkema et al. [22], was applied. ΔpH (interior alkaline) was determined from the fluorescence of pyranine [23] entrapped within the fused membranes or proteoliposomes. The reaction was performed at 25°C in a Perkin-Elmer MPF-4 spectrophotofluorimeter using excitation and emission wavelengths of 450 nm and 520 nm, respectively. Reaction mixtures contained (final concentrations) 10 mM Hepes/KOH pH 7.0, 45 mM KCl, 5 mM $MgCl_2$ and membranes with entrapped pyranine as indicated. After the addition of 10 μM cytochrome *c* to the membrane suspension, the reaction was initiated by the simultaneous addition of 20 mM ascorbate and 200 μM $Ph(NMe_2)_2$, unless indicated otherwise. Valinomycin and nigericin were used at final concentrations of 40 nM and 75 nM, respectively. Pyranine was entrapped by adding 200 μM pyranine to the membranes prior to freeze-thaw sonication. External pyranine was removed by chromatography of the membrane suspension over a Sephadex G-25 (course, 1 × 20 cm). Membranes with entrapped pyranine eluted in the void volume.

For calculation of $\Delta\psi$, the internal volume of the cytochrome *c* oxidase proteoliposomes was estimated to be 5 μl/mg phospholipid from the trapped amount of calcein [24]. A value of 8 μl/mg protein was used for the internal volume of the fused membranes, which was estimated from the trapped amount of calcein [24] and from the equilibration of [^{14}C]leucine under nonenergized conditions.

Transport assays

For K^+-diffusion potential-driven leucine accumulation, 6 μl of a concentrated membrane suspension (0.07–0.1 mg *S. cremoris* protein) loaded with potassium in the presence of 20 μM valinomycin, was rapidly diluted into 200 μl of 10 mM Hepes/NaOH pH 7.0, 45 mM NaCl, 20 mM methylamine, 5 mM $MgCl_2$ and 3.1 μM [U-^{14}C]leucine (12.4 TBq/mol). Methylamine was included in order to dissipate the everted ΔpH (interior acid) which is generated as a result of a $\Delta\psi$-induced proton flux across the membrane (A. J. M. Driessen, unpublished results). The magnitude of the imposed $\Delta\psi$ was varied by changing the external potassium and sodium concentrations.

For counterflow entrance, 2 μl of a concentrated membrane suspension (0.05 mg *S. cremoris* protein), loaded with 5 mM leucine (30 min, 20°C) was diluted into 100 μl 10 mM Hepes/KOH pH 7.0, 45 mM KCl, 5 mM $MgCl_2$, 40 nM nigericin, 2 μM valinomycin and [U-^{14}C]leucine, giving a final external concentration of 103 μM.

Leucine and alanine accumulation driven by a Δp generated by cytochrome *c* oxidase activity was performed by incubating the fused membranes (0.05 mg *S. cremoris* protein and 0.049 nmol oxidase) in 100 μl of 10 mM Hepes/KOH pH 7.0, 45 mM KCl, 5 mM $MgCl_2$, 10 mM ascorbate and 200 μM $Ph(NMe_2)_2$. Energization was initiated by the addition of 20 μM cytochrome *c*. After 0.5 min, [U-^{14}C]leucine (12.4 TBq/mol) or [U-^{14}C]alanine (43 TBq/mol) was added to a final concentration of 3.1 μM and 1.3 μM, respectively.

Transport was carried out at 25°C and was terminated by the addition of 2 ml ice-cold 100 mM LiCl, filtered on a 0.45-μM cellulose nitrate filter (Millipore) and washed once with 2 ml ice-cold 100 mM LiCl. Dried filters were transferred to scintillation vials containing 5 ml scintillation fluid and radioactivity was measured with a liquid scintillation counter (Packard Tri-Carb-460 CD, Packard Instruments Comp.).

Other analytical methods

Cytochrome *c* oxidase activity was measured with the spectroscopic assay [17] or polarographic assay as described. The orientation of cytochrome *c* oxidase was determined as described by Casey et al. [25]. Protein [26] and phospholipid phosphorus [27] were assayed as described.

Materials

Octyl β-D-glucopyranoside, crude asolectin and horse heart cytochrome *c* were purchased from Sigma Chemical Co. Asolectin was prepared from crude asolectin as described [28]. NBD-PE, Rh-PE and R_{18} were kindly provided by Dr J. Wilschut (Department of Physiological Chemistry, University of Groningen, The Netherlands). Pyranine was obtained from Eastman Kodak Co. ^{14}C-labeled amino acids were purchased from Amersham.

RESULTS

Fusion of Streptococcus cremoris *membrane vesicles with cytochrome* c *oxidase proteoliposomes*

Rapid freezing followed by a slow thawing and a brief sonication step has been applied for incorporating membrane proteins into tightly sealed liposomes [29, 30]. The technique has also been used for the phospholipid enrichment of bacterial [11, 31] and yeast membranes [32] and probably involves the process of membrane fusion [33]. However, the evidence for fusion is rather indirect and the results can in principle be explained by a unidirectional flow of phospholipids from the liposomes to the biological membranes. To test fusion between membrane vesicles derived from the lactic acid bacterium *S. cremoris* and proteoliposomes, containing beef heart mitochondrial cytochrome *c* oxidase, two independent fusion assays were applied which can demonstrate intermixing of the phospholipids of both membranes. The resonance energy transfer fusion assay [19] monitors the decrease in fluorescence energy transfer between fluorescent donor (NBD-PE) and acceptor (Rh-PE) phospholipids when both probes, originally incorporated into the liposomal membrane, dilute into the lipid surface area of a nonlabeled membrane [19]. Since both fluorescent phospholipid probes have been shown to be non-exchangable [20], a decrease of energy transfer can only occur by membrane fusion. The second method is based on the self-quenching properties of the fluorescent dye octadecyl rhodamine-β-chloride (R_{18}) [20] which can be inserted into biological membranes. Dilution of this probe in the plane of the nonlabeled membrane results in a relief of self-quenching, which is proportional to the extent of dilution [20]. Both assays have been extensively applied to study the kinetics of fusion between artificial phospholipid vesicles and biological membranes (for review see [34]) and their values for establishing the occurrence of membrane fusion are well recognized. In one set of experiments fusion between nonlabeled *S. cremoris* membrane vesicles and cytochrome *c* oxidase proteoliposomes labeled with 0.5 mol/100 mol phospholipid phosphorus of both NBD-PE and Rh-PE, was studied. In the second set of experiments, *S. cremoris* membrane vesicles were labeled with R_{18} (4 mol/100 mol phospholipid phosphorus) and fused with nonlabeled cytochrome *c* oxidase proteoliposomes. Both assays indicate similar extents of fusion at different ratios of *S. cremoris* membrane vesicles and cytochrome *c* oxidase proteoliposomes (Fig. 1, closed symbols). This figure also shows the

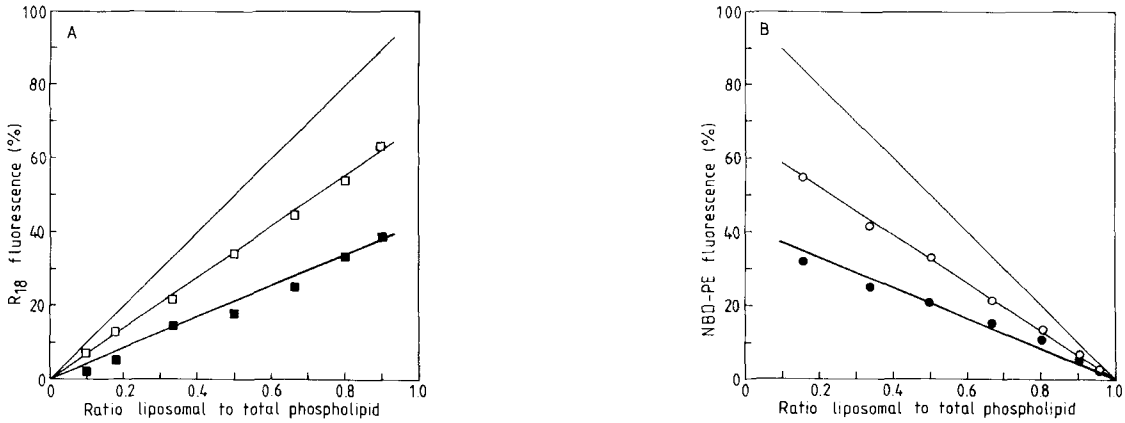

Fig. 1. *Fusion of* S. cremoris *membrane vesicles with cytochrome* c *oxidase proteoliposomes at different ratios of bacterial vesicles to proteoliposomes.* (A) Fusion of bacterial membranes labeled with 4 mol R_{18}/100 mol phospholipid phosphorus with nonlabeled proteoliposomes. (B) Fusion of bacterial membranes with proteoliposomes containing NBD-PE and Rh-PE at a final concentration of 0.6 mol/100 mol phospholipid phosphorus each. (■, ●) Fusion induced by freeze/thaw sonication; (□, ○) fusion induced by low pH (pH 4.0). The solid line represents the theoretically expected fluorescence intensity of the complete intermixing of all the phospholipids in the system, ignoring a possible effect of membrane proteins on the energy-transfer efficiency between NBD-PE and Rh-PE or on the self-quenching of R_{18}

theoretically expected fluorescence developments of the probe(s) distribute evenly over the *S. cremoris* membrane vesicles and cytochrome *c* oxidase proteoliposome, assuming that the surface area is only determined by the phospholipid content and ignoring a possible effect of membrane proteins on the fluorescence quantum yield of the fluorescent probes. Driessen et al. [10] have demonstrated that the maximal observed fluorescence development was about 40% lower than the theoretical maximum when bacterial membranes were fused with liposomes. Maximal fluorescence development was obtained when the membranes were fused at pH 4.0 [10] (Fig. 1, open symbols), indicating that complete fusion was achieved. These results demonstrate that both the freeze/thaw sonication and the low-pH-induced fusion procedure lead to a significant extent of fusion between both membranes. It should be emphasized that experiments described below were done with highly concentrated membrane preparations during the freeze/thaw sonication procedure, which facilitates aggregration of the membranes and subsequently may promote fusion.

Since the fused membranes obtained by the low-pH procedure have a relatively high ion permeability (see below), the freeze/thaw sonication procedure is more attractive for bioenergetic studies. The fused membranes were further examined using discontinous sucrose density gradients [14]. When *S. cremoris* membrane vesicles were mixed with cytochrome *c* oxidase proteoliposomes at alkaline pH (pH 8.0), the *S. cremoris* membrane vesicles were recovered as a single band with a buoyant density of 1.20 g/cm^3, while the proteoliposomes were found at a low density (1.09 g/cm^3). Upon freeze/thaw sonication, the fused membranes were recovered as a single band with an intermediate density and this density decreased with increasing ratio of proteoliposomes/*S. cremoris* membrane vesicles (Table 1). This band contained all the *S. cremoris* membrane proteins, liposomal phospholipids and cytochrome *c* oxidase activity. Although not shown, at the ratio used for all subsequent experiments (phospholipid ratio of bacterial membranes/proteoliposomes of 0.08) only about 5% of the proteoliposomes were recovered as a single band at their original density, while all the *S. cremoris* membrane vesicles were found in the band designated as fused membranes.

Table 1. *Effect of the ratio of cytochrome* c *oxidase proteoliposome/* S. cremoris *membrane vesicles on the buoyant density of the fused membranes*

Buoyant densities were estimated from the refractive index of the peak fractions on sucrose density gradients at 20 °C. The last two values were determined on a mixture of cytochrome *c* oxidase proteoliposomes and *S. cremoris* membrane vesicles at alkaline pH 8 without a fusion step

Oxidase/*S. cremoris* protein	Phospholipid/protein	Buoyant density at 20 °C
nmol/mg	μmol/mg	g/cm^3
0.92	8.5	1.114
0.52	6.0	1.121
0.21	3.3	1.129
0.07	1.7	1.153
0.04	1.3	1.184
Cytochrome oxidase proteoliposomes	21.3	1.094
S. cremoris membrane vesicles	0.8	1.204

Further evidence for fusion was obtained by freeze-fracture electron microscopy of the liposomal, bacterial and fused membranes [14]. Concomitant with the phospholipid enrichment, the intramembranous particle density of fused membranes decreased with respect to the particle density of the *S. cremoris* membrane vesicles, but the particle density remained higher than that of the liposomal membrane (data not shown).

Generation of a proton motive force in the fused membranes

The ability to generate a proton motive force in the fused membranes by cytochrome-*c*-oxidase-mediated oxidation of reduced cytochrome *c* was investigated. The magnitude of the $\Delta\psi$ generated was estimated from the uptake of the lipophilic cation tetraphenylphosphonium (Ph_4P^+) using a Ph_4P^+-selective electrode. A high-energy independent binding of

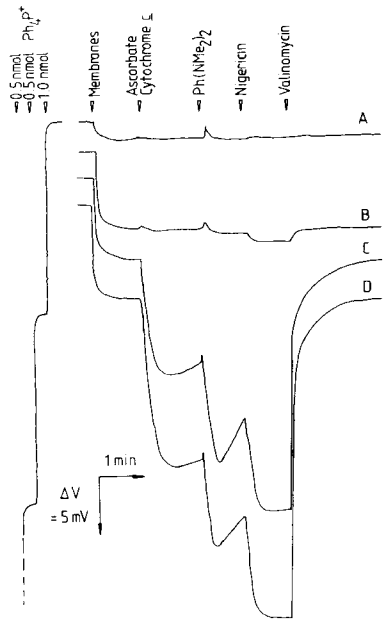

Fig. 2. *Tetraphenylphosphonium (Ph_4P^+) uptake by* S. cremoris *membrane vesicles alone (A), cytochrome* c *oxidase proteoliposomes alone (D) and by* S. cremoris *membrane vesicles fused with cytochrome* c *oxidase proteoliposomes by freeze/thaw treatment (B) followed by brief sonication (C). S. cremoris* membrane vesicles (0.24 mg protein) and proteoliposomes (0.23 nmol oxidase) were used in the medium described under Materials and Methods. Fused membranes were prepared as described under Materials and Methods. Ascorbate (10 mM), cytochrome *c* (20 μM), $Ph(NMe_2)_2$ (400 μM), nigericin (10 μM) and valinomycin (2 μM) were added as indicated

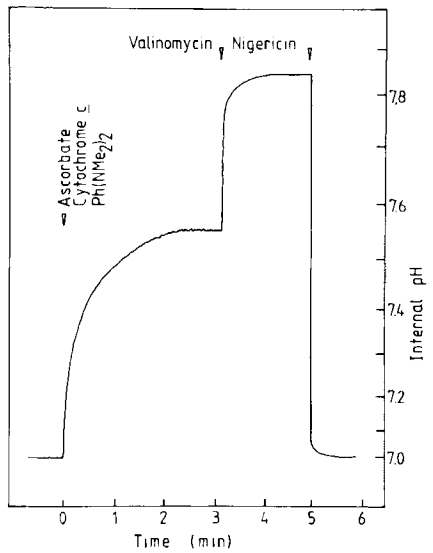

Fig. 3. *Internal pH change induced by the oxidation of reduced cytochrome* c *by* S. cremoris *membrane vesicles fused with cytochrome* c *oxidase proteoliposomes by freeze-thaw sonication.* Internal pH changes were measured by following the fluorescence of pyranine entrapped in the fused membranes as described under Methods. The reaction was initiated by addition of 10 μM cytochrome *c*, 200 μM $Ph(NMe_2)_2$ and 20 mM ascorbate. Valinomycin and nigericin were added sequentially to final concentrations of 40 nM and 75 nM, respectively as indicated. Fluorescence changes were calibrated by titration with 1 M KOH in the presence of nigericin. The reaction mixture contained fused membranes containing 0.12 mg *S. cremoris* membrane protein and 0.12 nmol cytochrome *c* oxidase in a final volume of 2 ml

Ph_4P^+ to the membranes occurred which could be considerably diminished by preparing the liposomes in the presence of calcium (5 mM). Binding was decreased further in the presence of magnesium ions or in high-ionic-strength buffer. When cytochrome *c* was added to the ascorbate-containing suspension of fused membranes, a rapid accumulation of Ph_4P^+ occurred followed by a slow release of accumulated Ph_4P^+ (Fig. 2, curve C). Addition of the electron-mediator $Ph(NMe_2)_2$ resulted in a further increase of Ph_4P^+ uptake. Stimulation of Ph_4P^+ uptake $Ph(NMe_2)_2$ was hardly observed when high ascorbate concentrations were used (50 mM). At increasing ascorbate or increased $Ph(NMe_2)_2$ concentrations the turnover of cytochrome *c* oxidase increased (data not shown). Concomitant with an increase of the ascorbate concentration, an increase in the ratio of reduced versus oxidized cytochrome *c* was observed. These observations indicate that electron transfer between ascorbate and oxidized cytochrome *c* is the rate-limiting step in the electron transfer to oxygen under the conditions employed. In the following experiments 20 mM ascorbate and 400 μM $Ph(NMe_2)_2$ were used as electron donors. As a result of sonication of the fused membranes for 4–8 s, the $\Delta\psi$ was significantly increased upon addition of the electron donors (compare curve B and C in Fig. 2). This is most probably due to a decrease in ion permeability since sonicated fused membranes have an increased capacity to maintain an artificially imposed ΔpH by the use of a nigericin-mediated potassium diffusion gradient (data not shown). The accumulation of Ph_4P^+ under optimum conditions was enhanced by the addition of low concentrations of the ionophore nigericin (10 nM) and a constant $\Delta\psi$ was reached. The effect of nigericin on Ph_4P^+ uptake was more pronounced at low external pH (5.5), which is consistent with the known ability of nigericin to collapse the ΔpH which subsequently can be compensated by an increase of the $\Delta\psi$. Addition of valinomycin, an ionophore which dissipates the $\Delta\psi$ since it mediates the influx of K^+, caused a rapid release of Ph_4P^+ to the energy-independent binding level. *S. cremoris* membrane vesicles did not accumulate Ph_4P^+ in the presence of ascorbate/$Ph(NMe_2)_2$/cytochrome *c* (Fig. 2, curve A) in contrast to cytochrome *c* oxidase proteoliposomes (Fig. 2, curve D). The finding that nigericin stimulated Ph_4P^+ uptake in the fused membranes provided indirect evidence that cytochrome-*c*-oxidase-mediated oxidation of reduced cytochrome *c* generated a significant ΔpH (interior alkaline) in addition to a $\Delta\psi$. Direct evidence for the generation of a ΔpH was obtained from internal pH measurements by monitoring the fluorescence of membrane vesicle-entrapped pyranine, an important, pH-sensitive fluorophore [23]. Upon addition of ascorbate/$Ph(NMe_2)_2$/cytochrome *c* to the fused membranes a significant alkalinization of the internal space was observed (Fig. 3). Valinomycin caused a further alkalinization while nigericin induced an immediate dissipation of the ΔpH. Similar results were obtained when the ΔpH was measured by flow dialysis using [^{14}C]acetate (data not shown). Upon addition of ascorbate/$Ph(NMe_2)_2$/cytochrome *c* to membrane vesicles of *S. cremoris* no internal pH change was found but the addition of this electron donor to cytochrome *c* oxidase proteoliposomes resulted in the generation of a significant ΔpH (not shown). Quantification of the Δp generated by cytochrome *c* oxidase in proteoliposomes and in fused membranes is given in Table 2. The valinomycin-induced conversion of a $\Delta\psi$ into a ΔpH was incomplete, while the nigericin-induced conversion of ΔpH into a $\Delta\psi$

Table 2. *Quantification of $\Delta\psi$ (interior negative) and ΔpH (interior alkaline) in fused membranes and cytochrome* c *oxidase proteoliposomes with different electron donors and electron mediators*
0.23 nmol oxidase was used for Δp measurements. Nigericin (Nig) and valinomycin (Val) were used at final concentrations of 40 nM and 20 nM, respectively

Electron donor	Cytochrome *c* (20 μM)	Ionophore	Fused membranes			Proteoliposomes		
			$\Delta\psi$	$-Z\Delta pH$	Δp	$\Delta\psi$	$-Z\Delta pH$	Δp
			mV			mV		
Ascorbate (20 mM)	+	Nig	− 90	0	− 90	− 59	0	− 59
Ascorbate (20 m) and $Ph(NMe_2)_2$ (400 μM)	+	−	− 97	− 30	− 127	− 62	− 36	− 98
		Val	0	− 51	− 51	0	− 58	− 58
		Nig	− 115	0	− 115	− 90	0	− 90
Ascorbate (20 mM) and phenazine methosulphate (10 μM)	−	Nig	− 61	0	− 61	− 39	0	− 39
	+	Nig	− 96	0	− 96	− 69	0	− 69

was essentially complete. It should be emphasized that these conversions were only observed when the ionophores were used at very low concentrations. In contrast to the cytochrome *c* oxidase proteoliposomes, no respiratory control could be detected in the fused membranes.

Orientation of cytochrome c *oxidase in the fused membranes*

About 55−60% of the cytochrome *c* oxidase molecules were oriented with their cytochrome *c* binding site located at the outer surface of the fused membranes. This orientation of cytochrome *c* oxidase in the fused membranes was slightly less asymmetric than in the proteoliposomes since in the fused membranes about 62−67% of the cytochrome *c* oxidase molecules had this orientation.

Proton-motive-force-driven solute transport in the fused membranes

The time course of leucine uptake in the fused membranes driven by an artificially imposed $\Delta\psi$ by means of a valinomycin-mediated potassium diffusion gradient is shown in Fig. 4. The uptake of both leucine and Ph_4P^+ (data not shown) proceeds in the fused membranes for a longer period of time than in the bacterial membranes (Fig. 4). In these latter membranes a transient uptake pattern was observed. These results indicate that fused membranes are tightly sealed structures with a relatively low ion permeability. In agreement with this conclusion was the observation that an artificially imposed ΔpH, generated by means of an outwardly directed acetate diffusion gradient, was maintained in the fused membranes for a longer period of time and consequently prolonged uptake of leucine was observed (data not shown). These results demonstrate that a functional leucine carrier was present in the fused membranes. The activity of this leucine carrier appeared to be hardly affected by the phospholipid enrichment of the membrane by the fusion process. The initial rate of counterflow entrance (inset of Fig. 4) at a leucine concentration of 100 μM was comparable to that observed in the nonfused membranes. The overshoot phenomenon observed with counterflow entrance furthermore is expected for a carrier-mediated leucine-uptake process. Similar observations were also made in fused membranes prepared from *S. cremoris* membrane vesicles and liposomes which did not contain cytochrome *c* oxidase.

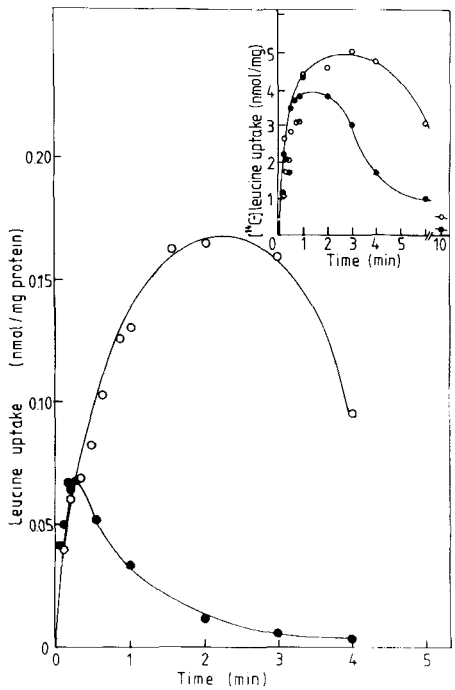

Fig. 4. *Leucine transport in* S. cremoris *membrane vesicles (●) and* S. cremoris *membrane vesicles fused with cytochrome* c *oxidase proteoliposomes by freeze/thaw sonication (○) driven by a valinomycin-induced K^+ diffusion potential of − 91 mV or by leucine counterflow (inset)*

The uptake of leucine in fused membranes which contain cytochrome *c* oxidase was energized by the oxidation of the electron donor system ascorbate/$Ph(NMe_2)_2$/cytochrome *c*. Under these conditions leucine was rapidly accumulated by the fused membranes (Fig. 5). The Δp ($\Delta\psi$ in the presence of nigericin) was about − 65 mV. This initial rate of uptake was comparable to the rate observed in the presence of a potassium diffusion potential of − 60 mV (see inset of Fig. 5). Unlike the potassium diffusion potential-driven leucine uptake, oxidase-driven leucine uptake continued for about 3 min and reached steady-state levels of accumulation.

It should be emphasized that leucine uptake coupled to cytochrome *c* oxidase activity was only observed in the fused membranes and not in the bacterial membranes nor in the cytochrome *c* oxidase proteoliposomes (data not shown).

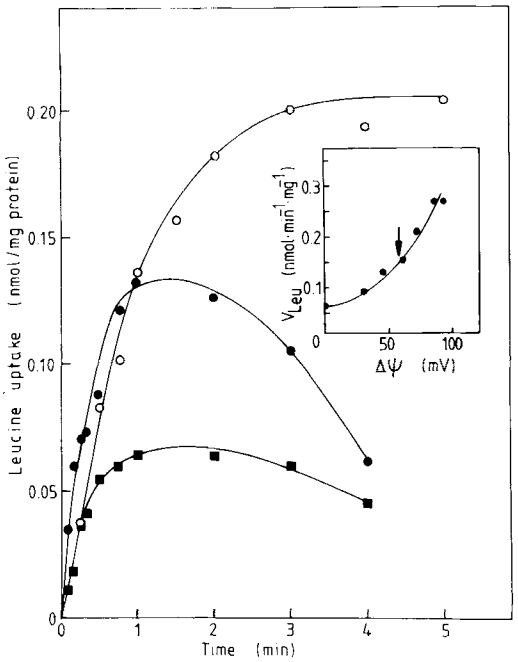

Fig. 5. *Leucine transport in* S. cremoris *membrane vesicles fused with cytochrome* c *oxidase proteoliposomes by freeze/thaw sonication driven by a valinomycin-induced K^+ diffusion potential of −86 mV (●) and −60 mV (■) or by the addition of ascorbate (20 mM), $Ph(NMe_2)_2$ (400 μM) and cytochrome* c *(20 μM) (□).* Inset; relation between the initial rate of leucine uptake (V_{Leu}) and the $\Delta\psi$, imposed by a K^+ diffusion potential. The initial rate of leucine uptake observed in the presence of ascorbate/$Ph(NMe_2)_2$ cytochrome *c* is indicated by an arrow

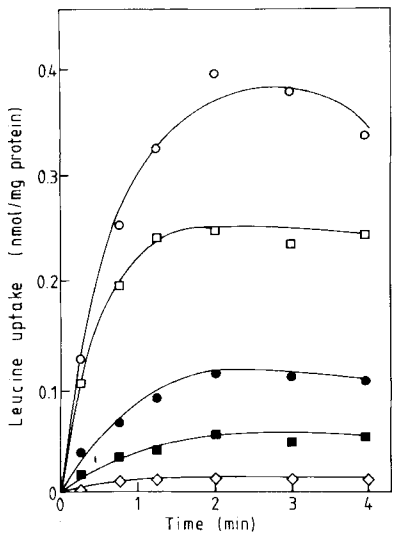

Fig. 6. *Leucine uptake by fused membranes induced by the addition of ascorbate/$Ph(NMe_2)_2$/cytochrome* c *(□), ascorbate/cytochrome* c *(●), oxidized cytochrome* c *(◇), ascorbate/phenazine methosulphate (■) and ascorbate/phenazine methosulphate/cytochrome* c *(□).* Where indicated by the symbols the reaction mixture contained 10 mM ascorbate, 100 μM phenazine methosulfate, 20 μM cytochrome *c* and/or 200 μM $Ph(NMe_2)_2$

When in the presence of cytochrome *c* the electron-mediator reduced phenazine methosulphate was used instead of $Ph(NMe_2)_2$, a slightly reduced leucine uptake level was observed (Fig. 6). Low activities were observed in the absence of cytochrome *c* with the electron donor system ascorbate/

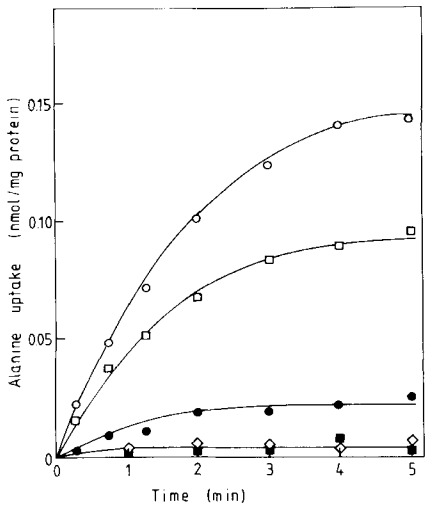

Fig. 7. *Effect of 1 mM cyanide (◇), 20 nM nigericin (□), 4 μM valinomycin (●) and 10 μM S-13 (■) on alanine transport in fused membranes in the presence of 10 mM ascorbate, $Ph(NMe_2)_2$ and 20 μM cytochrome* c *(□)*

phenazine methosulphate, while in the presence of the electron-donor system ascorbate/$Ph(NMe_2)_2$ or oxidized cytochrome *c* no uptake of leucine was detectable. The rate of leucine uptake observed in the presence of the various electron donors correlated well with the generated Δp (Table 2).

Leucine uptake was completely abolished by the cytochrome *c* oxidase inhibitor cyanide (1 mM). The addition of the uncoupler 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitro-salicylanilide (S-13) (10 μM) decreased leucine uptake to the level observed in the absence of electron donors [14]. These results show that the Δp generated by oxidase activity functions as a driving force for leucine uptake.

Alanine was accumulated to a similar extent as leucine but the initial rate of uptake was lower (Fig. 7). Valinomycin inhibited alanine uptake almost completely, while nigericin had only a slight inhibitory effect on alanine uptake (Fig. 7). These results are consistant with the Δp generated under those conditions (Table 2). No uptake of alanine was observed in the presence of cyanide (1 mM) or the uncoupler S-13 (10 μM).

Similar high transport activities were observed for the amino acids isoleucine, valine, alanine and serine in the fused membranes (data not shown).

DISCUSSION

This paper describes a relatively simple procedure for the functional incorporation of a Δp-generating system into biological membranes. The Δp-generating system used in this study is beef heart mitochondrial cytochrome *c* oxidase. Proteoliposomes containing this enzyme were fused with membrane vesicles derived from the homofermentative lactic acid bacterium *Streptococcus cremoris* by a freeze/thaw sonication procedure. Fusion was demonstrated by: (a) intermixing of membrane phospholipids; (b) the buoyant density of the membranes after fusion was between those of the liposomes and the bacterial membranes [14]; (c) the intramembranous particle density was within the density of the starting membranes observed on the external fracture surface of the

membranes as visualized by freeze-fracture electron microscopy [14]; and (d) transport of amino acids coupled to the generation of a Δp by cytochrome *c* oxidase activity.

After freeze/thaw treatment the fused membranes are highly aggregated. A brief sonication step is necessary to obtain a membrane preparation in which a Δp of considerable magnitude can be generated in the presence of the electron donor system ascorbate/$Ph(NMe_2)_2$/cytochrome *c*. This effect is due to a decrease in ion permeability of the fused membranes rather than activation of cytochrome *c* oxidase. This is evident from the increased capacity of the fused membranes with respect to the *S. cremoris* membrane vesicles to maintain an artificially generated $\Delta\psi$ and ΔpH. Similar to fused membranes obtained by the freeze-thaw procedure, low-pH-induced fusion gives rise to the formation of membranes with a relatively high ion permeability. However, with that procedure an additional sonication step or extensive vortexing also yields tightly sealed membrane vesicles (A. J. M. Driessen, unpublished results). Although the bacterial membranes were enriched with a large amount of exogenous phospholipids (endogenous phospholipid was about 8% of total phospholipid), the activity of the carrier protein for leucine was hardly affected. These characteristics make the hybrid liposome/bacterial membrane vesicle system obtained with this fusion procedure almost ideal for studies on the role of Δp in solute transport. This is demonstrated by the observation that the Δp generated by cytochrome *c* oxidase activity functions as a driving force for the uptake of several amino acids in the fused membranes.

The attractive feature of using cytochrome *c* oxidase as a Δp-generating system is that it provides a model system in which exclusively a right-side-out oriented Δp, e.g. interior negative and alkaline, can be generated. Such a unidirectional Δp generation has not been obtained with bacteriorhodopsin, despite many attempts to incorporate this protein into a right-side-out orientation (A. J. M. Driessen, unpublished results).

The results presented show that the incorporation procedure of cytochrome *c* oxidase into biological membranes offers attractive possibilities for the energization of membrane vesicles of organisms which lack a suitable Δp-generating system. This includes membrane vesicles derived from fermentative bacteria and membrane vesicles derived from the cell or organelle membrane of eukaryotic cells. Furthermore the hybrid liposome/biological membrane vesicles offers attractive possibilities for the study of systems in which anomalies of the bulk-phase chemiosmosis are observed.

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